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Contribution of the liver to the pathogenesis of feline infectious peritonitis

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Abstract

Feline infectious peritonitis (FIP) is a well-known and widely distributed coronavirus (FCoV)-induced systemic disease in cats. It is characterised by fibrinous-granulomatous serositis with protein-rich effusions into body cavities, granulomatous phlebitis and periphlebitis, and granulomatous inflammatory lesions in several organs. The development of FIP lesions is triggered by activated, virus infected monocytes. Many systemic changes, including recurrent fever, are seen. These all indicate excessive cytokine release, the source of which has yet to be determined. This study investigated the possible contribution of the liver to the disease by examining it for the transcription and translation of relevant cytokines. A quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) for the detection of feline interleukin-1β (IL-1β), IL-6, IL-10, IL-12p40, and tumour necrosis factor-α (TNF-α) was carried out on liver samples of naturally and experimentally infected cats with FIP, and FCoV-infected SPF cats without FIP. Cytokine transcription was detected in all groups, however mRNA levels were significantly higher for IL-6 in all cats with FIP; in those with naturally acquired FIP, all other cytokines were also upregulated. Immunohistology confirmed cytokine translation and identified the hepatocytes as the main source. These results show a potential contribution by hepatocytes to systemic effects such as fever and vasculitis in the development of FIP.

Key words: Cytokine release; hepatocytes; liver; feline coronavirus, feline infectious peritonitis

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Zusammenfassung

Die Feline Infektiöse Peritonitis (FIP) ist eine tödliche Coronavirus-induzierte Erkrankung der Katzen, die durchgranulomatöse Serositiden mit proteinreichen Ergüssen sowie granulomatöse (Peri)-Phlebitiden und Entzündungsprozessen in Organen charakterisiert ist. Entscheidend für die Erkrankung sind aktivierte, Virus-infizierte Monozyten. Das Auftreten zahlreicher systemische Veränderungen, wie rezidivierendes Fieber, aufspricht dafür, dass es ausserdem zu einer übermässigen Zytokinfreisetzung kommt; deren Quelle ist bisher jedoch nicht bekannt.
Die vorliegende Arbeit hat den möglichen Beitrag der Leber (unabhängig vom Vorhandensein typischer FIP-Läsionen) am Krankheitsgeschehen untersucht. Eine quantitative reverse Transkription-Polymerasekettenreaktion zum Nachweis von felinem Interleukin-1β (IL-1β), IL-6, IL-10, IL-12p40 und Tumornekrosefaktor-α (TNF-α) an Leberproben von natürlich erkrankten und experimentell infizierten Katzen mit FIP sowie FCoV-infizierten SPF-Katzen ohne FIP. wies eine Transktiption dieser Zytokine in der Leber aller Tiere nach. Bei Katzen mit FIP war die IL-6-Transkription signifikant erhöht, bei den natürlich infizierten Tieren gilt dies auch für alle anderen Zytokine. Mittels Immunhistologie wurde die Translation der Zytokine bestätigt und die Expression vor allem durch Hepatozyten nachgewiesen. Diese Ergebnisse deuten darauf hin, dass die Leber bei der Entstehung der systemischen FIP-Veränderungen wie Fieber und Vaskulitis eine Rolle spielt.

Stichwörter: Zytokinproduktion; Hepatozyten; Leber; Felines Coronavirus; Feline Infektiöse Peritonitis

Original Article

Contribution of the liver to the pathogenesis of feline infectious peritonitis.

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Abstract

Feline infectious peritonitis (FIP) is a widespread and almost invariably fatal systemic disease of cats induced by feline coronavirus (FCoV). The classical lesions consist of pyogranulomatous-necrotising phlebitis and periphlebitis of multiple organs, often accompanied by protein-rich effusions into body cavities. The development of FIP lesions is triggered by activated, virus infected monocytes, whereas systemic changes such as recurrent fever indicate excessive systemic cytokine release, the source of which has yet to be determined. This study investigated the possible contribution of the liver to the disease by examining it for the transcription and translation of relevant cytokines. A quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) for the detection of feline interleukin-1β (IL-1β), IL-6, IL-10, IL-12p40, and tumour necrosis factor-α (TNF-α) was carried out on liver samples of cats with FIP and FCoV-infected cats without FIP. Cytokine transcription was detected in both groups. However, mRNA levels were significantly higher in the cats with FIP. Immunohistology confirmed cytokine translation and identified hepatocytes as the main source. These results show a potential contribution by hepatocytes to systemic effects such as fever and vasculitis in the development of FIP.

Key words: Cytokine release; Feline coronavirus; Feline infectious peritonitis; Hepatocytes; Liver

Introduction

Feline infectious peritonitis (FIP) is a coronavirus-induced fatal systemic immune-mediated disease in cats, characterised by fibrinous-granulomatous serositis, often with protein-rich effusions into body cavities, granulomatous phlebitis and periphlebitis, and granulomatous inflammatory lesions in several organs (Hayashi et al., 1977; Weiss and Scott, 1981a; Kipar et al., 1998; Kipar et al., 2005). The disease presents clinically with recurrent fever and signs reflecting the distribution of organ lesions. The development of FIP lesions is triggered by activated, virus infected monocytes. These induce the hallmark of the disease, a granulomatous phlebitis that occurs in a range of organs (Kipar et al., 2005). When the granulomatous phlebitis is present, animals also exhibit generalised activation of venous endothelial cells which, together with other systemic changes such as fever, indicate excessive systemic cytokine release (Kipar et al., 2005; Dinarello, 2007).

Pro-inflammatory cytokines are the main mediators of the innate immune response, allowing communication between and priming of the various components of the innate immune system, e.g. activation of leukocytes and endothelial cells (reviewed by Dinarello, 2007; Akdis et al., 2011).

IL-1, IL-6, and TNF-α are of particular relevance in the feline acute phase response (Paltrinieri, 2008). In FIP, their role does not appear consistent. Goitsuka et al. (1988, 1990) found high IL-6 and IL-1 activity in sera, ascitic fluid, and the supernatant of cultured peritoneal exudate cells. IL-6 mRNA levels in peripheral blood mononuclear cells (PBMC) were found to be unaltered, mildly increased or variable (Gunn-Moore et al., 1998; Kiss et al., 2004; Gelain et al., 2006), whilst IL-10 and IL-12 transcription was markedly depressed (Gunn-Moore et al., 1998). In an experimental feline coronavirus (FCoV) vaccination study, IL-12, TNF-α, and, less intensely, IL-10 mRNA levels increased after challenge independent of the development of FIP (Kiss et al., 2004). In contrast, a study on natural FIP cases found no elevation in serum

TNF-α and IL-1 transcription levels at the time of euthanasia (Gelain et al., 2006).

More recent in vitro studies showed that virulent FCoV (i.e. FIPV)-infected macrophages produce TNF-α which in turn functions both as a possible contributor to lymphocyte apoptosis (observed in FIP (Haagmans et al., 1996; Kipar et al., 2001a)) and a stimulator of aminopeptidase N, the receptor for type II FCoV. Thereby, monocytes/macrophages likely enhance their own infection rate (Takano et al., 2007a, 2007b). However, in vivo FIP is associated with a decrease in TNF-α transcription in lymphatic tissues (Kipar et al., 2006b). This was seen in conjunction with increased IL-1β and IL-6 but decreased IL-12 and IL-10 transcription (Kipar et al., 2006b). Taken together, those previous studies suggest that other cell types are involved in the disease process. In cats with FIP, acute phase proteins produced by the liver such as haptoglobin and serum amyloid A, and in particular alpha$_1$-acid glycoprotein (AGP), have been shown to be elevated and are often considered key elements of the diagnostic process (Duthie et al., 1997; Giordano et al., 2004). In conjunction with appropriate clinical signs, raised AGP levels are more effective than effusion analysis or serology at discriminating between FIP and other diseases (Giori et al., 2011). This suggests more extensive hepatic involvement in the disease.

The liver is known as a major target for cytokines (Moshage, 1997; Ramadori and Armbrust, 2001) but a number of studies involving human, rat or murine hepatocytes have shown that these cells also produce cytokines, including IL-1, IL-6, IL-10, IL-12, and TNF-α (González-Amaro et al., 1994;Alfrey et al., 1995; Saad et al., 1995; Frede et al., 1996; Panesar et al., 1999; Stonāns et al., 1999; Dikopoulos et al., 2004). A preliminary immunohistological study indicated that in cats with FIP hepatocytes express TNF-α (unpublished data).

We therefore hypothesised that the liver also contributes to FIP via cytokine production. To evaluate this we measured relative mRNA levels of IL-1β, IL-6, IL-10, IL-12p40, and TNF-α

by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and used immunoblotting and immunohistology to confirm translation and identify the cells producing the cytokines.

Methods

Animals and tissue processing

The study was undertaken on three groups of cats. Group 1 comprised 16 cats with FIP including 12 pet cats (5 months to 2 years) submitted for diagnostic necropsy that had died or were euthanased with natural FIP (Group 1a). A further four cats had been euthanased with FIP after experimental intra-peritoneal infection with the serotype I FCoV strain FIPV-UCD at the University of Utrecht, The Netherlands (Group 1b). Approval for this experiment was obtained from the Ethical Committee of Utrecht University (approval number: 0502.0802, 10.10.2005). All showed clinical signs of FIP which necessitated euthanasia of two cats at 3.5 and 4 weeks post infection (p.i.) whilst the remaining two were euthanased at the end of the experiment (11 weeks p.i.).

For Group 1, the diagnosis of FIP was confirmed by gross, histological, and immunohistological examination (Kipar et al., 1998). Cases were split into wet, dry, and mixed forms of the disease based on clinical and necropsy findings.

Group 2 consisted of 14 clinically healthy, male, specific pathogen free (SPF) cats euthanased between 14 and 80 days p.i. (Meli et al., 2004) that had been perorally infected at an age of 8.5 to 27 weeks with a previously isolated serotype I FCoV field strain of low virulence (feline enteric coronavirus; FECV) (Meli et al., 2004). This experiment was performed under the Swiss regional legislation (project license number TVB 66/2000; date of

approval: 31.07.2001). All had tested positive for CoV antibodies and circulating FCoV-specific immune complexes and were confirmed to be systemically FCoV infected by the presence of a monocyte-associated FCoV viraemia.

All animals were necropsied within 30 min of death. Liver samples from grossly normal regions were collected and immediately frozen at -80 °C for RNA extraction, or fixed in 10% buffered formalin for 24-48 h and routinely paraffin wax embedded for histological and immunohistological examination.

The third group comprised six healthy untreated SPF cats, aged 36-38 months, that had been euthanased at the University of Glasgow, UK as part of a study performed under UK Home Office Project Licence PPL 60/3735; approved 04.01.2008. From these cats, formalin fixed, paraffin embedded liver samples were kindly provided by Prof M Hosie.

Reverse transcription and quantitative polymerase chain reaction (qRT-PCR) for feline cytokines

From each frozen liver sample, approximately 100 mg was taken for RNA extraction and subsequent cDNA synthesis, following previously published protocols (Kipar et al., 2006a). qRT-PCR for feline IL-1β, IL-6, IL-10, IL-12p40, and TNF-α was carried out on the cDNA samples, using previously published assays (Table 1a; (Leutenegger et al., 1999; Kipar et al., 2001b, 2006b)). The levels of the housekeeping gene feline glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal controls. All reactions were run in duplicate, using identical and previously published amplification conditions and assay compositions (Kipar et al., 2001b, 2006b).

Relative quantification of cytokine transcripts and statistical analysis

Relative quantification of cytokine signals was done by the comparative C_T method and is reported as the n-fold difference relative to the fGAPDH cDNA levels for each sample. This serves to normalise for differences in the amount of total nucleic acid added to each reaction and the efficiency of the reverse transcriptase step as previously described (Kipar et al., 2001a, 2006a). The programme Minitab was used for statistical comparison; a two-tailed Mann-Whitney test was applied at the 95 % confidence level on the premise that cytokine levels in the two groups were different. First the natural FIP (Group 1a) cases were compared with the experimental FIP (Group 1b) cases to determine if they could be classed as one group. As no significant difference was found between these subgroups, all of Group 1 was compared to Group 2. Both Group 1a and 1b were also compared separately to Group 2.

Within Group 1, cytokine mRNA levels from cats with the wet, dry or mixed form were compared.

The IL-12p40:IL-10 mRNA ratio was evaluated using the Independent Samples Median Test.

Antibodies specific for feline cytokines

A panel of rabbit anti-peptide antibodies, specific for feline IL-1β, IL-6, IL-10, IL-12p40, and TNF-α was commercially produced (Genosphere Biotechnologies, Paris, France). Antibodies were raised against the C-terminus of the proteins by using 14 amino acid long synthetic peptides with an additional cysteine residue at the N-terminus coupled to keyhole limpet hemocyanin (KLH) carrier (Table 1b).

All antisera had been determined by the supplier by ELISA to have a titre greater than 1:10,000 when tested against the peptide antigen.

Antibody specificity was confirmed by ELISA and dot blot, using recombinant cytokines (recombinant human IL-1β and TNF-α (Peprotech); feline IL-6 (Serotec); feline IL-1β, TNF-α, IL-12 (R&D Systems)), following routine protocols. The antibodies were subsequently used in immunoblots and immunohistology.

Immunoblots for the detection of feline cytokines in the liver

Frozen liver samples (100 - 400 mg) from Group 1b were pulverised at 2,000 rpm for 1 min in a liquid nitrogen cooled stainless steel chamber according to manufacturer's recommendations (Braun Mikrodismembranator) and dissolved in 2 ml/g lysis buffer (Takeuchi et al., 2005) for 30 min at 4 °C. After further centrifugation (3,000 *g*, 10 min at 4 °C), the supernatant was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions following routine protocols to confirm the presence of proteins of appropriate size.

Immunoblotting was performed with the recombinant cytokines over a range of concentrations (total loading of 0.625 – 100 ng), in addition to the liver samples. The liver samples were separated in SDS-PAGE, followed by transfer onto nitrocellulose according to standard protocols. After transfer the membrane was incubated in 0.005 % hydrogen peroxide in phosphate-buffered saline (PBS) for 30 min to block endogenous peroxidase activity (liver homogenates) and in blocking buffer (3 % w/v skimmed milk powder in PBS) for 1 h, followed by 15 - 18 h incubation with the primary anti-feline cytokine antibodies (1:500 to 1:10,000 in PBS). To confirm that the secondary antibody was not binding feline immunoglobulins present

in the liver sample, it was incubated with cat serum prior to its application to the membrane (negative controls).

Histopathological examination and immunohistology for feline cytokines

Formalin-fixed, paraffin embedded liver sections (3 - 5 µm) from animals of each group were routinely stained with haematoxylin and eosin for histopathological examination, and a selection subjected to immunohistology for feline cytokines using the peroxidase anti-peroxidase (PAP) method (Kipar et al., 1998), with diaminobenzidine as the chromogen and Papanicolaou's haematoxylin counterstaining (five cats from Group 1a and all four cats from Group 1b).

Sections incubated with rabbit pre-immune serum and/or PBS instead of the primary antibody and sections incubated with antibodies blocked by the respective peptide served as negative controls.

Various dilutions and reaction conditions had been tested and assessed histologically; they were deemed appropriate when no non-specific staining of collagen and nuclei was seen.

Sections were assessed independently by two pathologists and staining was graded in a semi-quantitative manner as negative, weak, moderate or strong, based on the number of positive hepatocytes and average staining intensity.

Results

Cytokine transcription in the liver

All tested cytokines were detectable in both FCoV-infected cats with and without FIP (Groups 1 and 2), and of these in the majority of animals (81 – 100 % depending on the cytokine, Table 2).

Relative transcription levels of all studied cytokines varied between animals in both groups, but were significantly higher ($P < 0.05$) in cats with FIP. However, they also exhibited higher variance between individual cats in this group (Fig. 1). Interestingly, the within group variation in transcription levels for each cytokine was lower in Group 1b than in Group 1a, and so were the transcription levels overall (though not significantly so). Therefore, when comparing Group 2 with Group 1b alone, a significant difference was only observed for IL-6 (Fig. 1). Similar trends within Group 1 were also observed in the IL-12p40:IL-10 mRNA ratio. The ratio was overall significantly higher in Group 1 than Group 2 ($P = 0.047$), and indeed IL-10 levels were higher than IL-12 in all Group 2 cats, whereas in half of the Group 1a cats they were lower. Group 1b cats were in between, with slightly higher IL-10 levels in all animals.

An assessment of medians between Group 1 and 2 for each cytokine found the smallest quantitative difference for IL-1β and IL-10; mRNA levels being only ~10 fold higher in Group 1. For IL-12 and TNF-α a close to 100 fold increase was seen. The difference was largest for IL-6, the mRNA level of which was nearly 1,000 fold higher in cats with FIP. Interestingly, IL-6 was also the cytokine that was transcribed at a lower level than all other cytokines in Group 2 (though not statistically significant) whereas it was on a par with the others in Group 1. IL-6 was also the only cytokine that varied between the disease form; its transcription was significantly higher ($P = 0.04$; Mann Whitney) in cats with wet FIP than in those with the dry or mixed form.

Cytokine translation in the liver

Immunoblots performed on Group 1b liver homogenates yielded bands consistent with the expected proteins for IL-10 (both an 18kD monomer and the 36kD glycosylated homodimer (Tan et al., 1993), IL-12 (both the p40 monomer and the active p30p45 heterodimer (Brunda, 1994)), and TNF-α (26 kDa non-glycosylated membrane form and the 51 kDa pyramidal trimer (Corti et al., 1992; Otto et al., 1997)).

Immunohistology was used to identify the cells producing the cytokines. The SPF cat livers served to assess FCoV-independent constitutive protein expression. Alongside this, all livers were examined for any histopathological changes and FIP-specific inflammatory processes (Kipar et al., 1998).

In SPF cat livers were histologically unaltered. Cytokine expression was mainly evident in the bile duct epithelium which exhibited variable expression of all cytokines (Fig. 2). There was also occasional evidence (i.e. in one or two animals for each cytokine) of very low level expression by hepatocytes, represented by a weak, finely granular cytoplasmic staining (Fig. 2G, I). Furthermore, Kupffer cells in all SPF cats occasionally expressed IL-1β and in two and one cat respectively also IL-6 and IL-12 (Fig. 2G).

The livers of FCoV-infected cats without FIP generally exhibited mild portal lymphocyte infiltration as well as occasional small clusters of lymphocytes and scattered individual neutrophils within the sinusoids. The cytokine expression pattern and intensity was similar to that seen in SPF cats; hepatocytes largely exhibited either no or very weak staining.

In cats with FIP, the majority of livers exhibited typical lesions, i.e. fibrinosuppurative perihepatitis and/or focal to multifocal granulomatous-necrotising lesions. Cytokine expression by inflammatory cells was generally weak, but all cytokines were found to be expressed by neutrophils and macrophages as well as low numbers of Kupffer cells (Fig. 2H). Hepatocytes were found to express all cytokines. This was seen either as a diffuse staining of all hepatocytes

(Fig. 2F, H, J) or appeared variable in its intensity between individual cells (Fig. 2B, D). The semi-quantitative assessment of cytokine expression and its relation to the transcription levels did not provide any evidence of a direct correlation.

Sections stained with pre-immune sera or PBS in place of the primary antibody did not yield any reaction.

Discussion

The present study is the first to focus on the expression of cytokines in the liver, and in particular by hepatocytes, in cats suffering from a systemic inflammatory disease, FIP. The panel of cytokines was chosen as FIP is driven by activated monocytes and characterised by vascular inflammatory lesions and fever.

Using a combination of methods to demonstrate cytokine transcription and in situ protein expression, we showed that the feline liver produces a range of pro-inflammatory and immunomodulatory cytokines, and identified the hepatocytes as a source of these.

In FIP, the hepatic transcription of IL-1β, IL-6, IL-10, IL-12 and TNF-α was significantly upregulated. Interestingly, when comparing natural and experimental FIP, the range of the natural cases extended higher. It is possible that the prior SPF status of the experimentally infected cats accounts for this as they would not have had previous exposure to circulating FCoVs and/or other common infectious pathogens.

All cytokines appeared to be constitutively expressed also in the liver of SPF cats, where immunohistology identified bile duct epithelial cells as the main source, with Kupffer cells

contributing as well. In cats with FIP, inflammatory cells within the typical macrophage dominated lesions were also found to express cytokines. However, these likely played a minor role in the overall hepatic expression, since we extracted RNA from tissue specimens without grossly visible FIP lesions and generally found immunohistological evidence of more consistent cytokine expression by hepatocytes than by inflammatory cells. The immunohistological staining results did not allow an accurate quantification of translation. Neither could we identify a direct correlation between mRNA and protein levels. One explanation for both may be the different sensitivities of the two methods. Another is the manifold levels of pre- and posttranslational regulation to which cytokines are exposed. Taking TNF-α alone, there is regulation of RNA at export, post-transcriptional and translational, with multiple mechanisms at each stage (Giambelluca et al., 2013). However, our objective was to view in situ cytokine expression in cells of the liver. Our results provide strong evidence that hepatocytes play a role in the pathogenesis of FIP. This is supported by data from a mouse study showing that hepatocytes can produce at least as much IL-6 per cell as macrophages (Panesar et al., 1999). Our study did not assess plasma levels of the cytokines so we did not confirm that they enter the general circulation but it is likely since other proteins produced by hepatocytes, such as APPs, do so and exert a systemic effect (Moshage, 1997).

IL-1β, IL-6 and TNF-α are the major inflammatory cytokines the release of which from the liver in FIP could contribute to the induction of lesions and clinical signs, such as fever, capillary leakage and hepatic APP production (Dinarello et al., 1986; Moshage, 1997; Dinarello, 2007). Also, the hypoalbuminaemia and weight loss seen in FIP (Paltrinieri et al., 1998; de Groot-Mijnes et al., 2005; Paltrinieri, 2008) could be due to IL-1β induced decreased albumin production to allow for greater APP synthesis, and TNF-α-induced muscle breakdown.

IL-6, known to be particularly stimulated by IL-1β and TNF-α (Kent et al., 1998), showed the greatest upregulation of the studied cytokines in association with FIP. In wet FIP, significant IL-6 activity has been detected in sera and peritoneal exudate cells (Goitsuka et al., 1990). The wet, dry, and mixed forms of FIP indicate different disease states with a variable degree of vascular permeability (Takano et al., 2011; Pedersen, 2014). IL-6 can induce vascular endothelial growth factor (VEGF) production and pulmonary vascular permeability (Loeffler et al., 2005; Feurino et al., 2007; Gurkan et al., 2011), and the degree of ascites was found to be correlated with serum VEGF levels in cats with FIP (Takano et al., 2011), This could explain, via VEGF upregulation, the particularly high hepatic IL-6 transcription levels seen in cats with wet FIP in our study. Hepatic IL-6 release could also contribute to the progressive plasma cell infiltration seen in older FIP lesions (Kipar et al., 1998; Kipar & Meli, 2014), as IL-6 induces the final maturation of B cells into plasma cells (Kishimoto, 1989).

TNF-α and IL-1β induce expression of adhesion molecules and chemokine production by endothelial cells (EC) which induces leukocyte binding (Abbas et al., 2012). In line with this, upregulation of β_2-integrins was seen on leukocytes from cats with FIP (Olyslaegers et al.., 2013). Concurrently, TNF-α, IL-1β and IL-6 can also mediate the firm binding of monocytes to EC and can be expected to contribute to the destruction of the vascular basal lamina seen in FIP phlebitis (Hayashi et al., 1977; Watanabe et al., 1993; Kipar et al., 1998; Kipar et al., 2005), as they upregulate matrix metalloproteinase secretion from monocytes (Sarén et al., 1996; Robinson et al., 2002).

The effects of IL-1β include pyrogenicity, stimulation of B cell antibody production, endothelial retraction and gap formation, and neutrophilia; again all likely key to the pathogenesis of FIP (Weiss and Scott, 1981b; Staruch and Wood, 1983; Dinarello, 1996). Interestingly, IL-1β has been shown experimentally to more than double the half-life of IL-6

mRNA, and TNF-α had a similar, but less intense effect (Iwasaki et al., 2011). Accordingly, IL-1β could have two relevant synergistic effects in FIP; stimulating IL-6 transcription and prolonging the lifespan of IL-6 mRNA (Kent et al., 1998), thereby potentiating its effects.

IL-10, is an anti-inflammatory and immunosuppressive cytokine (de Waal Malefyt et al., 1991; Buchwald et al., 1999) that inhibits inflammatory cytokine transcription by monocytes (de Waal Malefyt et al., 1991), but also complements IL-6 as it can stimulate antibody production and, at high concentrations, increases the phagocytic capacity of monocytes/macrophages (Buchwald et al., 1999). Increased viral load in monocytes is seen as one of the key steps in the development of FIP (Kipar et al., 2006a; Kipar and Meli, 2014) and as the virus is capable of inducing IL-1 β and TNF-α in vitro (Goitsuka et al., 1988; Takano et al., 2007b) this may contribute to increased production of these inflammatory cytokines and the ensuing systemic effects.

We also found IL-12 upregulation in the liver in association with FIP. The pro-inflammatory effects of IL-12 are usually counteracted by IL-10 which attempts to return the immune response to homeostatic base levels (Cao et al., 2002). The IL-12:IL-10 ratio has been used as a measure of the balance between pro- and anti-inflammatory states in a number of disease conditions (Watson et al., 2012). In our study we found an overall higher hepatic IL-12:IL-10 mRNA ratio in FIP than in healthy FCoV-infected cats. Despite being archetypally pro-inflammatory, at high concentrations IL-12 can inhibit the immune response and the generation of cytotoxic T lymphocytes (Lasarte et al., 1999; Lee et al., 2000). Via interferon-γ-mediated Fc receptor upregulation it could induce enhanced macrophage infection (Puddu et al., 1997; Mortola et al., 1998). In contrast to the liver, the mesenteric lymph nodes were previously found to downregulate IL-12 transcription in FIP (Kipar et al., 2006). It would appear that neither high

IL-12 nor high IL-10 are themselves protective and indeed plasmids encoding IL-12 have been found to enhance susceptibility to disease in FIPV vaccination studies (Glansbeek et al., 2002).

Conclusions

This is the first functional study of its kind on the liver in FIP and its potential role in the manifestation of disease. Hepatocytes are able to produce inflammatory and immune-modulatory cytokines (IL-1β, -6, -10, -12, and TNF-α) and these are transcribed at significantly higher levels in cats with FIP than in healthy FCoV infected cats. This may provide a large amplifying step in a cascade of cytokine release in FIP.

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Conflict of interest

None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

References

Abbas, A.K., Lichtman, A.H., Pillai, S., 2012. Cellular and Molecular Immunology, 7th ed. Saunders Elsevier.

Akdis, M., Burgler, S., Crameri, R., Eiwegger, T., Fujita, H., Gomez, E., Klunker, S., Meyer, N., O'Mahony, L., Palomares et al., 2011. Interleukins, from 1 to 37, and interferon-γ: receptors, functions, and roles in diseases. Journal of Allergy and Clinical Immunology 127, 701–721.

Alfrey, E.J., Most, D., Wang, X., Lee, L.K., Holm, B., Krieger, N.R., Sibley, R.K., Huie, P., Dafoe, D.C., 1995. Interferon-gamma and interleukin-10 messenger RNA are up-regulated after orthotopic liver transplantation in tolerant rats: evidence for cytokine-mediated immune dysregulation. Surgery 118, 399–404; discussion 404–405.

Brunda, M., 1994. Interleukin-12. Journal of Leukocyte Biology 55, 280–288.

Buchwald, U.K., Geerdes-Fenge, H.F., Vöckler, J., Ziege, S., Lode, H., 1999. Interleukin-10: effects on phagocytosis and adhesion molecule expression of granulocytes and monocytes in a comparison with prednisolone. European Journal of Medical Research 4, 85–94.

Cao, S., Liu, J., Chesi, M., Bergsagel, P.L., Ho, I.-C., Donnelly, R.P., Ma, X., 2002. Differential regulation of IL-12 and IL-10 gene expression in macrophages by the basic leucine zipper transcription factor c-Maf fibrosarcoma. Journal of Immunology 169, 5715–5725.

Corti, A., Fassina, G., Marcucci, F., Barbanti, E., Cassani, G., 1992. Oligomeric tumour necrosis factor alpha slowly converts into inactive forms at bioactive levels. Biochemical Journal 284, 905–910.

de Groot-Mijnes J.D., van Dun J.M., van der Most R.G., de Groot R.J., 2005. Natural history of a recurrent feline coronavirus infection and the role of cellular immunity in survival and disease. Journal of Virology 79, 1036–1044.

De Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C.G., de Vries, J.E., 1991. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. Journal of Experimental Medicine 174, 1209–1220.

Dikopoulos, N., Wegenka, U., Kröger, A., Hauser, H., Schirmbeck, R., Reimann, J., 2004. Recently primed CD8+ T cells entering the liver induce hepatocytes to interact with naïve CD8+ T cells in the mouse. Hepatology 39, 1256–1266.

Dinarello, C.A, 2007. Historical insights into cytokines. European Journal of Immunology 37 Suppl 1, S34–S45.

Dinarello, C.A., 1996. Biologic basis for interleukin-1 in disease. Blood 87, 2095–147.

Dinarello, C.A., Cannon, J.G., Wolff, S.M., Bernheim, H.A., Beutler, B., Cerami, A., Figari, I.S., Palladino, M.A., Connor, J.V.O., 1986. Tumor necrosis factor (cachectin) is an

endogenous pyrogen and induces production of interleukin 1. Journal of Experimental Medicine 163, 1433–1450.

Duthie, S., Eckersall, P.D., Addie, D.D., Lawrence, C.E., Jarrett, O., 1997. Value of alpha 1-acid glycoprotein in the diagnosis of feline infectious peritonitis. Veterinary Record 141, 299–303.

Feurino, L.W., Zhang, Y., Bharadwaj, U., Zhang, R., Li, F., Fisher, W.E., Brunicardi, F.C., Chen, C., Yao, Q., Min, L., 2007. IL-6 stimulates Th2 type cytokine secretion and upregulates VEGF and NRP-1 expression in pancreatic cancer cells. Cancer Biology and Therapy 6, 1096–1100.

Frede, S., Fandrey, J., Jelkmann, W., 1996. Interleukin 1 beta and phorbol ester induce tumour necrosis factor alpha production in a hepatic cell line (HepG2). Pflügers Archiv: European Journal of Physiology 431, 923–927.

Gelain, M.E., Meli, M., Paltrinieri, S., 2006. Whole blood cytokine profiles in cats infected by feline coronavirus and healthy non-FCoV infected specific pathogen-free cats. Journal of Feline Medicine and Surgery 8, 389–399.

Giambelluca, M.S., Laflamme, C., Pouliot, M., 2013. Post-transcriptional regulation of tumour necrosis factor alpha biosynthesis: Relevance to the pathophysiology of rheumatoid arthritis. OA Inflammation 1, 1–6.

Giordano, A., Spagnolo, V., Colombo, A., Paltrinieri, S., 2004. Changes in some acute phase protein and immunoglobulin concentrations in cats affected by feline infectious peritonitis or exposed to feline coronavirus infection. Veterinary Journal 167, 38–44.

Giori, L., Giordano, A, Giudice, C., Grieco, V., Paltrinieri, S., 2011. Performances of different diagnostic tests for feline infectious peritonitis in challenging clinical cases. Journal of Small Animal Practice 52, 152–157.

Glansbeek, H.L., Haagmans, B.L., te Lintelo, E.G., Egberink, H.F., Duquesne, V., Aubert, A., Horzinek, M.C., Rottier, P.J.M., 2002. Adverse effects of feline IL-12 during DNA vaccination against feline infectious peritonitis virus. Journal of General Virology 83, 1–10.

Goitsuka, R., Ohashi, T., Ono, K., Yasukawa, K., Koishibara, Y., Fukui, H., Ohsugi, Y., Hasegawa, A., 1990. IL-6 activity in feline infectious peritonitis. Journal of Immunology 144, 2599–2603.

Goitsuka, R., Onda, C., Hirota, Y., Hasegawa, A., Tomoda, I., 1988. Feline interleukin 1 production induced by feline infectious peritonitis virus. Japanese Journal of Veterinary Science 50, 209–214.

González-Amaro, R., García-Monzón, C., García-Buey, L., Moreno-Otero, R., Alonso, J.L., Yagüe, E., Pivel, J.P., López-Cabrera, M., Fernández-Ruiz, E., Sánchez-Madrid, F., 1994. Induction of tumor necrosis factor alpha production by human hepatocytes in chronic viral hepatitis. Journal of Experimental Medicine 179, 841–848.

Gunn-Moore, D. A, Caney, S.M., Gruffydd-Jones, T.J., Helps, C.R., Harbour, D. A, 1998. Antibody and cytokine responses in kittens during the development of feline infectious peritonitis (FIP). Veterinary Immunology and Immunopathology 65, 221–242.

Gurkan, O.U., He, C., Zielinski, R., Rabb, H., King, L.S., Dodd-o, J.M., D'Alessio, F.R., Aggarwal, N., Pearse, D., Becker, P.M., 2011. Interleukin-6 mediates pulmonary vascular permeability in a two-hit model of ventilator-associated lung injury. Experimental Lung Research 37, 575–584.

Haagmans, B.L., Egberink, H.F., Horzinek, M.C., 1996. Apoptosis and T-cell depletion during feline infectious peritonitis. Journal of Virology 70, 8977–8983.

Hayashi, T., Goto, N., Takahashi, R., Fujiwara, K., 1977. Systemic vascular lesions in feline infectious peritonitis. Japanese Journal of Veterinary Science 39, 365–377.

Iwasaki, H., Takeuchi, O., Teraguchi, S., Matsushita, K., Uehata, T., Kuniyoshi, K., Satoh, T., Saitoh, T., Matsushita, M., Standley, D.M. et al. 2011. The IκB kinase complex regulates the stability of cytokine-encoding mRNA induced by TLR-IL-1R by controlling degradation of regnase-1. Nature Immunology 12, 1167–1175.

Kent, L.W., Rahemtulla, F., Hockett, R.D., Gilleland, R.C., Michalek, S.M., 1998. Effect of lipopolysaccharide and inflammatory cytokines on interleukin-6 production by healthy human gingival fibroblasts. Infection and Immunity 66, 608–614.

Kipar, A., Bellmann, S., Kremendahl, J., Köhler, K., Reinacher, M., 1998. Cellular composition, coronavirus antigen expression and production of specific antibodies in lesions in feline infectious peritonitis. Veterinary Immunology and Immunopathology 65, 243–257.

Kipar, A., Köhler, K., Leukert, W., Reinacher, M., 2001a. A comparison of lymphatic tissues from cats with spontaneous feline infectious peritonitis (FIP), cats with FIP virus infection but no FIP, and cats with no infection. Journal of Comparative Pathology 125, 182–191.

Kipar, A., Leutenegger, C.M., Hetzel, U., Akens, M.K., Mislin, C.N., Reinacher, M., Lutz, H., 2001b. Cytokine mRNA levels in isolated feline monocytes. Veterinary Immunology and Immunopathology 78, 305–315.

Kipar, A., May, H., Menger, S., Weber, M., Leukert, W., Reinacher, M., 2005. Morphologic features and development of granulomatous vasculitis in feline infectious peritonitis. Veterinary Pathology 42, 321–330.

Kipar, A., Baptiste, K., Barth, A., Reinacher, M., 2006a. Natural FCoV infection: cats with FIP exhibit significantly higher viral loads than healthy infected cats. Journal of Feline Medicine and Surgery 8, 69–72.

Kipar, A., Meli, M.L., Failing, K., Euler, T., Gomes-Keller, M. A, Schwartz, D., Lutz, H., Reinacher, M., 2006b. Natural feline coronavirus infection: differences in cytokine patterns in association with the outcome of infection. Veterinary Immunology and Immunopathology 112, 141–155.

Kipar, A., Meli, M.L., 2014. Feline infectious peritonitis: still an enigma? Veterinary Pathology 51, 505–526.

Kishimoto, T., 1989. The biology of interleukin-6. Blood 74, 1–10.

Kiss, I., Poland, a M., Pedersen, N.C., 2004. Disease outcome and cytokine responses in cats immunized with an avirulent feline infectious peritonitis virus (FIPV)-UCD1 and challenge-exposed with virulent FIPV-UCD8. Journal of Feline Medicine and Surgery 6, 89–97.

Lasarte, J.J., Corrales, F.J., Casares, N., López-Díaz de Cerio, A., Qian, C., Xie, X., Borrás-Cuesta, F., Prieto, J., 1999. Different doses of adenoviral vector expressing IL-12 enhance or depress the immune response to a coadministered antigen: the role of nitric oxide. Journal of Immunology 162, 5270–5277.

Lee, K., Overwijk, W.W., O'Toole, M., Swiniarski, H., Restifo, N.P., Dorner, A.J., Wolf, S.F., Sturmhoefel, K., 2000. Dose-dependent and schedule-dependent effects of interleukin-12 on antigen-specific CD8 responses. Journal of Interferon and Cytokine Research 20, 589–596.

Leutenegger, C.M., Mislin, C.N., Sigrist, B., Ehrengruber, M.U., Hofmann-Lehmann, R., Lutz, H., 1999. Quantitative real-time PCR for the measurement of feline cytokine mRNA. Veterinary Immunology and Immunopathology 71, 291–305.

Loeffler, S., Fayard, B., Weis, J., Weissenberger, J., 2005. Interleukin-6 induces transcriptional activation of vascular endothelial growth factor (VEGF) in astrocytes in vivo and regulates VEGF promoter activity in glioblastoma cells via direct interaction between STAT3 and Sp1. International Journal of Cancer 115, 202–213.

Meli, M., Kipar, A., Müller, C., Jenal, K., Gönczi, E., Borel, N., Gunn-Moore, D., Chalmers, S., Lin, F., Reinacher, M., Lutz, H., 2004. High viral loads despite absence of clinical and pathological findings in cats experimentally infected with feline coronavirus (FCoV) type I and in naturally FCoV-infected cats. Journal of Feline Medicine and Surgery 6, 69–81.

Mortola, E., Endo, Y., Mizuno, T., Ohno, K., Watari, T., Tsujimoto, H., Hasegawa, A., 1998. Effect of interleukin-12 and interleukin-10 on the virus replication and apoptosis in T-cells infected with feline immunodeficiency virus. Journal of Veterinary Medical Science 60, 1181–1185.

Moshage, H., 1997. Cytokines and the hepatic acute phase response. Journal of Pathology 181, 257–266.

Olyslaegers, D. A J., Dedeurwaerder, A., Desmarets, L.M.B., Vermeulen, B.L., Dewerchin, H.L., Nauwynck, H.J., 2013. Altered expression of adhesion molecules on peripheral blood leukocytes in feline infectious peritonitis. Veterinary Microbiology 166, 438–449.

Otto, C.M., Niagro, F., McGraw, R. A, Rawlings, C. a, 1997. Production of polyclonal antibodies to feline tumor necrosis factor. Clinical and diagnostic laboratory immunology 4, 487–90.

Paltrinieri, S., 2008. The feline acute phase reaction. Veterinary Journal 177, 26–35.

Paltrinieri, S., Cammarata, M.P., Cammarata, G., Comazzi, S., 1998. Some aspects of humoral and cellular immunity in naturally occuring feline infectious peritonitis. Veterinary Immunology and Immunopathology 65, 205–220.

Panesar, N., Tolman, K., Mazuski, J.E., 1999. Endotoxin stimulates hepatocyte interleukin-6 production. Journal of Surgical Research 85, 251–258.

Pedersen, N.C., 2014. An update on feline infectious peritonitis: Virology and immunopathogenesis. Veterinary Journal 201, 123–132.

Puddu, P., Fantuzzi, L., Borghi, P., Varano, B., Rainaldi, G., Guillemard, E., Malorni, W., Nicaise, P., Wolf, S.F., Belardelli F.et al.,1997. IL-12 induces IFN-gamma expression and secretion in mouse peritoneal macrophages. Journal of Immunology 159, 3490–3497.

Ramadori, G., Armbrust, T., 2001. Cytokines in the liver. European Journal of Gastroenterology and Hepatology 13, 777–784.

Robinson, S.C., Scott, K.A., Balkwill, F.R., 2002. Chemokine stimulation of monocyte matrix metalloproteinase-9 requires endogenous TNF-alpha. European Journal of Immunology 32, 404–412.

Saad, B., Frei, K., Scholl, F.A., Fontana, A., Maier, P., 1995. Hepatocyte-derived interleukin-6 and tumor-necrosis factor alpha mediate the lipopolysaccharide-induced acute-phase response and nitric oxide release by cultured rat hepatocytes. European Journal of biochemistry 229, 349–355.

Sarén, P., Welgus, H.G., Kovanen, P.T., 1996. TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages. Journal of Immunology 157, 4159–4165.

Staruch, M.J., Wood, D.D., 1983. The adjuvanticity of interleukin 1 in vivo. Journal of Immunology 130, 2191–2194.

Stonāns, I., Stonāne, E., Russwurm, S., Deigner, H.P., Böhm, K.J., Wiederhold, M., Jäger, L., Reinhart, K., 1999. HepG2 human hepatoma cells express multiple cytokine genes. Cytokine 11, 151–156.

Takano, T., Hohdatsu, T., Hashida, Y., Kaneko, Y., Tanabe, M., Koyama, H., 2007a. A “possible” involvement of TNF-alpha in apoptosis induction in peripheral blood lymphocytes of cats with feline infectious peritonitis. Veterinary Microbiology 119, 121–131.

Takano, T., Hohdatsu, T., Toda, A., Tanabe, M., Koyama, H., 2007b. TNF-alpha, produced by feline infectious peritonitis virus (FIPV)-infected macrophages, upregulates expression of type II FIPV receptor feline aminopeptidase N in feline macrophages. Virology 364, 64–72.

Takano, T., Ohyama, T., Kokumoto, A., Satoh, R., Hohdatsu, T., 2011. Vascular endothelial growth factor (VEGF), produced by feline infectious peritonitis (FIP) virus-infected monocytes and macrophages, induces vascular permeability and effusion in cats with FIP. Virus Research 158, 161–168.

Takeuchi, S., Goto, T., Mikami, K.-I., Miura, K., Ohshima, S., Yoneyama, K., Sato, M., Shibuya, T., Watanabe, D., Kataoka, E. et al., 2005. Genipin prevents fulminant hepatic failure resulting in reduction of lethality through the suppression of TNF-alpha production. Hepatology Research 33, 298–305.

Watanabe, H., Nakanishi, I., Yamashita, K., Hayakawa, T., Okada, Y., 1993. Matrix metalloproteinase-9 (92 kDa gelatinase/type IV collagenase) from U937 monoblastoid cells: correlation with cellular invasion. Journal of Cell Science 104, 991–999.

Watson, D.C., Sargianou, M., Panos, G., 2012. Interleukin-12 (IL-12)/IL-10 ratio as a marker of disease severity in Crimean-Congo hemorrhagic fever. Clinical and Vaccine Immunology 19, 823–824.

Weiss, R.C., Scott, F.W., 1981a. Pathogenesis of feline infectious peritonitis: pathologic changes and immunofluorescence. American Journal of Veterinary Research 42, 2036–2048.

Weiss, R.C., Scott, F.W., 1981b. Pathogenesis of feline infectious peritonitis: nature and development of viremia. American Journal of Veterinary Research 42, 382–390.

Table 1. A. Sequence of PCR primers and TaqMan probes specific for feline cytokines. A. Amino acid sequences used for anti-feline cytokine antibody production.

A.

Cytokine	Primers 5' – 3'	Probe
GAPDH[1] 82bp	57f: GCCGTGGAATTTGCCGT 138r: GCCATCAATGACCCCTTCAT	77p: CTCAACTACATGGTCTACATGTTCCAGTATGA TTCCA
IL-1β[2] 76bp	55f: AATGACCTGTTCTTTGAGGCTGAT 130r: CCAGAAAACTGTGGCTCAGGTT	84p: CGAAAAGATGAAGGGCAGCCTCCAA
IL-6[2] 110bp	132f: CCCTGCAGACAAAATGGAAGA 241r GTGCCTCCTTGCTGTCCTCA	181p: TGTTATAGTTGTCACACATCTCCTTTTTCAGT GCAGA
IL-10[1] 76bp	182f: TGCACAGCATATTGTTGACCAG 257r ATCTCGGACAAGGCTTGGC	209p: ACCCAGGTAACCCTTAAGGTCCTCCAGCA
IL-12p40[1] 81bp	253f: TGGCTTCAGTTGCAGGTTCTT 333r: TGGACGCTATTCACAAGCTCA	283p: CGGTTTGATGATGTCCCTGATGAAGAAGCT
TNF-α[2] 74bp	239f: CTTCTCGAACTCCGAGTGACAAG 312r: CCACTGGAGTTGCCCTTCA	283p: CGGTTTGATGATGTCCCTGATGAAGAAGCT

[1] Leutenegger et al., 1999; [2]Kipar et al., 2001b.

B.

Cytokine	NCBI Acc.-No.	Position	Amino acid sequence
IL-1β	AAA30814	251-264	Cys-NTKGGQDITDFIME
IL-6	P41683	191-204	Cys-LRRLEDFLQFSLRA
IL-10	AAC64708	144-157	Cys-STFSKLQEKGVYKA
IL-12p40	AAB93835	185-198	Cys-RDYKKYTVECQEGS
TNF	AAA30818	202-215	Cys-LEKGDRLSAEINLP

Table 2. Detection of cytokine mRNA transcription in the livers of naturally and experimentally infected cats with feline infectious peritonitis (FIP), and of experimentally feline coronavirus (FCoV)-infected, healthy cats (FCoV-infected cats without FIP) and results of statistical examinations (*P* values of a two-tailed Mann-Whitney comparison between different groups).

Cytokine	Group	Group 1 and 2 distribution statistics				Group comparison (*P* values; * = significant at 95 % CI)			
		No.+ve†	Median ($2^{-\Delta CT}$)	Mean ($2^{-\Delta CT}$)	SE	1a vs. 1b	1 vs. 2	1a vs. 2	1b vs. 2
IL-1β	1	15	0.00381	0.01280	0.00595	0.1014	0.0065*	0.0011*	0.8734
	2	14	0.00021	0.00066	0.00041				
IL-6	1	14	0.00540	0.05200	0.04640	0.7616	0.0004*	0.0005*	0.0144*
	2	12	0.00002	0.000002	0.000001				
IL-10	1	13	0.00375	0.01068	0.00370	0.1619	0.0357*	0.0327*	0.3667
	2	14	0.00045	0.00055	0.00011				
IL-12p40	1	15	0.00405	0.00749	0.00272	0.1296	0.0014*	0.0024*	0.0631
	2	14	0.00010	0.00013	0.00004				
TNF-α	1	14	0.00678	0.00755	0.00222	0.0785	0.0168*	0.0043*	0.8734
	2	13	0.00021	0.00034	0.00009				

No. +ve: number of cases with positive qRT-PCR result; SE: standard error; CI: confidence interval; Group 1: all cats with FIP, *n* = 16; Group 1a: naturally infected cats with FIP (*n* = 12); Group 1b: experimentally infected cats with FIP (*n* = 4); Group 2: FCoV infected cats without FIP (*n* = 14); †: All four Group 1b cases were positive for all tested cytokines.

Figures

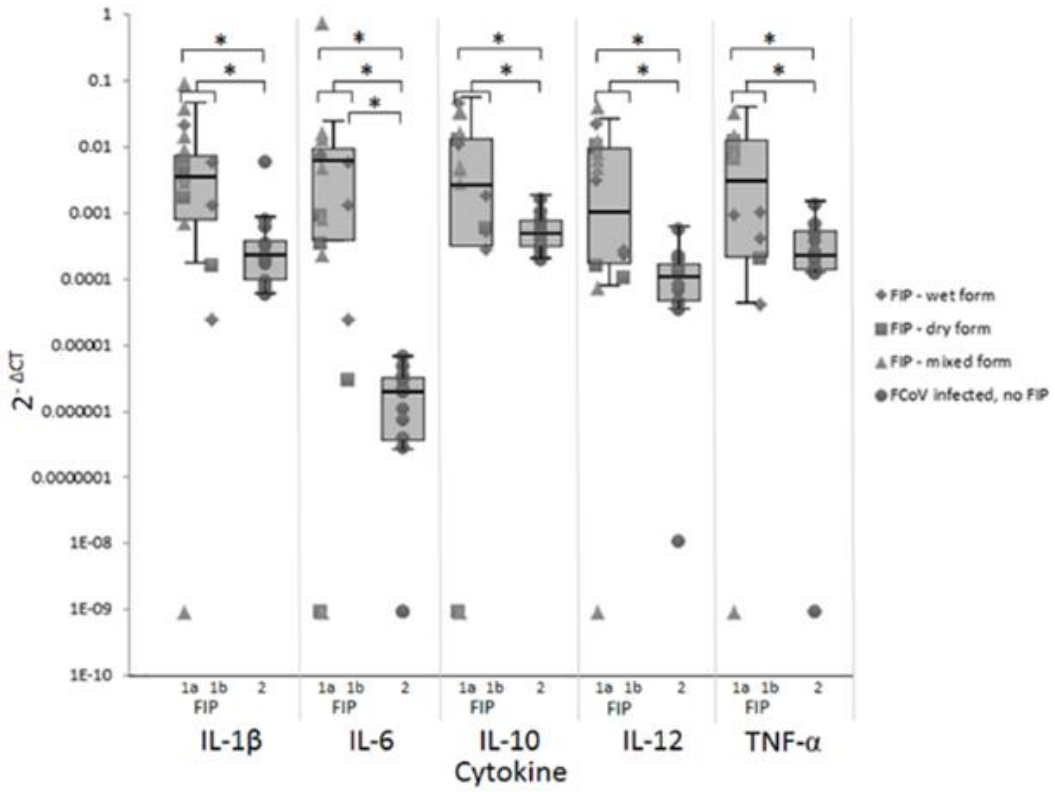

Fig. 1. Comparison of relative cytokine transcription levels between cats with feline infectious peritonitis (FIP) and healthy, feline coronavirus (FCoV)-infected cats; box and whisker plots together with illustration of disease form of individual cats. Group 1a - natural FIP cases; 1b - experimentally infected cats with FIP; 2 - FCoV-infected cats without FIP; * - statistically significant difference ($P < 0.05$).

Boxes indicate the median value and the interquartile range, whilst whiskers indicate the spread of values with the exception of outliers (calculated by SPSS as >1.5 box lengths).

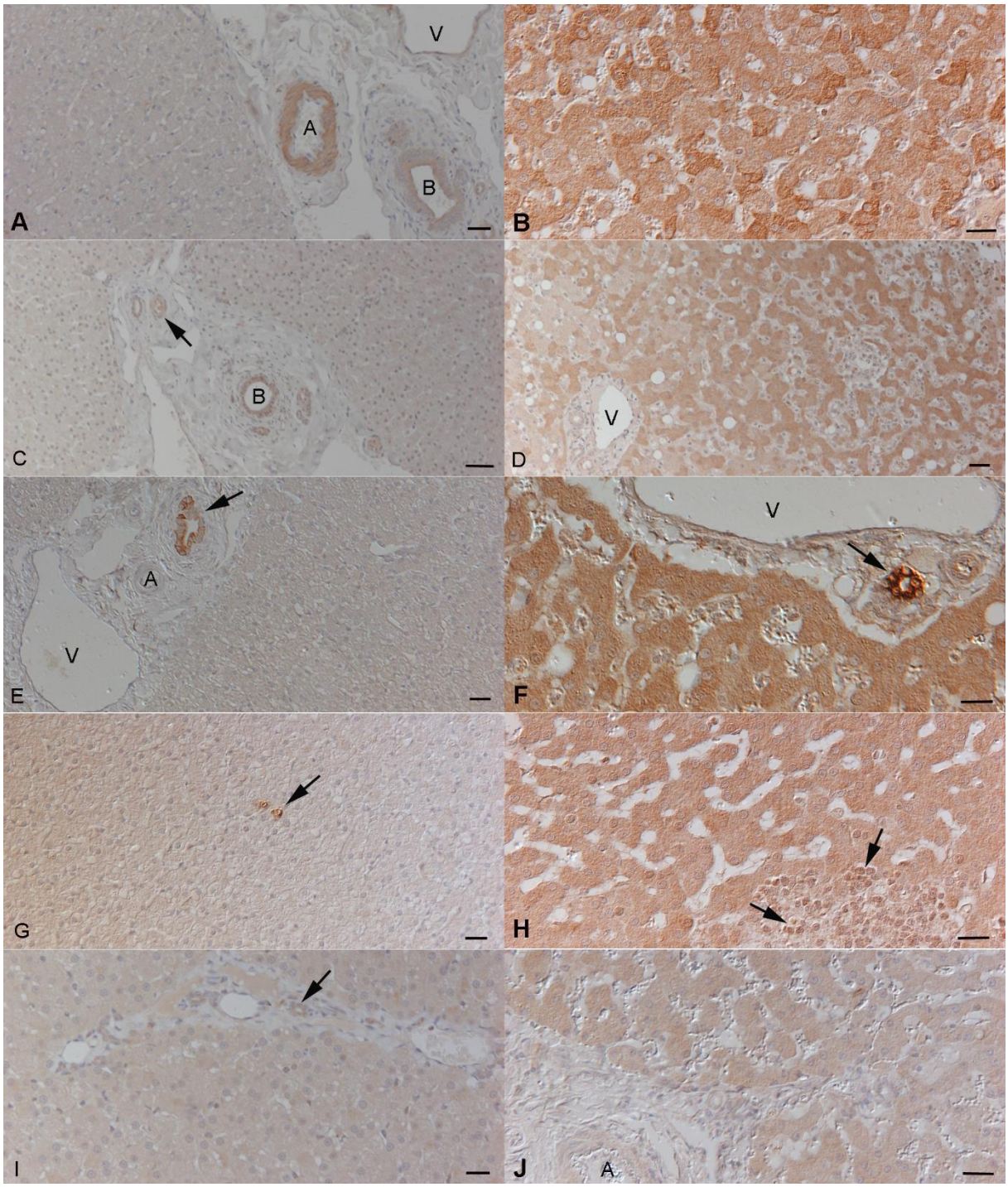

Fig. 2. Immunohistological staining for the expression of cytokines in the liver. Left column: specific pathogen free (SPF) cats; right column: cats with FIP. **A, B**: Expression of IL-1β. **A**: In SPF cats, expression is restricted to bile duct (B) epithelial cells and the media of small arteries (A). V: vein in portal area. **B**: In a FIP cat, hepatocytes exhibit variably intense expression. **C, D**: Expression of IL-6. **C**: In SPF cats, expression is restricted to a weak staining

in bile duct (B) epithelial cells and the media of small arteries (A). **D**: In a FIP cat, a large proportion of hepatocytes exhibit variably intense expression. V: vein in portal area. **E, F**: Expression of IL-10. **E**: In SPF cats, expression is restricted to bile duct epithelial cells (arrow). A: artery, V: vein in portal area. **F**: In a FIP cat, hepatocytes exhibit a diffuse strong expression. The staining in bile duct epithelial cells (arrow) is even stronger. V: vein in portal area. **G, H**: Expression of IL-12p40. **G**: In a SPF cat, very weak expression is seen within hepatocytes. Occasional positive Kupffer cells (arrow) are also seen. **H**: In a FIP cat, hepatocytes exhibit a diffuse weak to moderate expression. In a granulomatous infiltrate, there are some weakly positive macrophages (arrows). **I, J**: Expression of TNF-α. **I**: In a SPF cat, very weak expression is seen within hepatocytes and bile duct epithelial cells (arrow). **J**: In a cat with FIP, hepatocytes exhibit a diffuse weak staining. Peroxidase anti-peroxidase method, Papanicolaou's haematoxylin counterstain. Magnifications: C x 100; A, D, E, G, I x 200; B, F, H, J x 400.

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